

6395707

Detailed Description Text - DETX (52):

Also included within the scope of mutants herein are so-called glyco-scan mutants. This embodiment takes advantage of the knowledge of so-called glycosylation sites. Thus, where appropriate such a glycosylation site can be introduced so as to produce a species containing glycosylation moieties at that position. Similarly, an existing glycosylation site can be removed by mutation so as to produce a species that is devoid of glycosylation at that site. It will be understood, again, as with the other mutations contemplated by the present invention, that they are introduced within the so-called KDR and/or FLT-1 domains in accord with the basic premise of the present invention, and they can be introduced at other locations outside of these domains within the overall molecule so long as the final product does not differ in overall kind from the properties of the mutation introduced in one or both of said two binding domains.

5332671

Detailed Description Text - DETX (4):

Analogues or variants are defined as molecules in which the amino acid sequence, glycosylation, or other feature of native VEGF has been modified covalently or noncovalently. Thus, variants may or may not have a molecular weight of approximately 45 kD (as determined by SDS-PAGE carried out in the absence of a reducing agent such as, e.g., .beta.-mercaptoethanol or dithiothreitol). For example, unglycosylated VEGF having the native mature sequence will have a lower molecular weight on non-reducing SDS-PAGE. Amino acid sequence variants include not only alleles of the FIG. 2 sequence, but also predetermined mutations thereof. Generally, amino acid sequence variants have an amino acid sequence with at least about 80% homology, and more typically at least about 90% homology, to that of the native VEGF of FIG. 2, including variants with at least 95% homology, such as the human sequence shown in FIG. 10. Henceforth, the term VEGF shall mean either the native sequence or a variant form unless otherwise appropriate.

10/15/03

5219739

Detailed Description Text - DETX (23):

Biologically active vascular endothelial cell growth factor is produced in accordance with the teachings of this invention, as a homodimeric molecule. In this context, the term "homodimeric" refers to a dimer in which the two subunits have the same primary amino acid structure. As previously indicated, one or both of the subunits may be modified by N-linked glycosylation or neither of the subunits modified. A fully active protein is produced by expression and/or recovery of the polypeptide sequence encoded by the DNA sequence of the invention under conditions which allow the formation of disulfide bonds in order to form a dimer.

6090621

Detailed Description Text - DETX (20):

Any "polypeptide" of the invention, including a native or altered SIP polypeptide, includes any part of the protein including the mature protein, and further include truncations, variants, alleles, analogs and derivatives thereof. Variants can be spliced variants expressed from the same gene as the related protein. Unless specifically mentioned otherwise, such a polypeptide possesses one or more of the bioactivities of the protein, including for example protease activity, or inhibition of a protease. This term is not limited to a specific length of the product of the gene. Thus, polypeptides that are identical or contain at least 60%, preferably 70%, more preferably 80%, and most preferably 90% homology to the target protein or the mature protein, wherever derived, from human or nonhuman sources are included within this definition of a polypeptide. Also included, therefore, are alleles and variants of the product of the gene that contain amino acid substitutions, deletions, or insertions. The amino acid substitutions can be conservative amino acid substitutions or substitutions to eliminate non-essential amino acid residues such as to alter a glycosylation site, a phosphorylation site, an acetylation site, or to alter the folding pattern by altering the position of the cysteine residue that is not necessary for function, etc. Conservative amino acid substitutions are those that preserve the general charge, hydrophobicity/hydrophilicity and/or steric bulk of the amino acid substituted, for example, substitutions between the members of the following groups are conservative substitutions: Gly/Ala, Val/Ile/Leu, Asp/Glu, Lys/Arg, Asn/Gln, Ser/Cys/Thr and Phe/Trp/Tyr. Analogs include peptides having one or more peptide mimics, also known as peptoids, that possess the target protein-like activity. Included within the definition are, for example, polypeptides containing one or more analogs of an amino acid (including, for example, unnatural amino acids, etc.), polypeptides with substituted linkages, as well as other modifications known in the art, both naturally occurring and nonnaturally occurring. The term "polypeptide" also does not exclude post-expression modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations, myristoylations and the like.

5783416

Drawing Description Text - DRTX (17):

As indicated above, the SP variant of the invention may be provided in non-glycosylated form. This may, for instance, be accomplished by substituting Asn15 by another amino acid, e.g. Asp or Glu, or by substituting Thr17 by another amino acid except Ser, e.g. Ala. It is more likely, however, that one or more additional glycosylation sites will be introduced into this domain, for instance by replacing Arg10 by Asn, Gly 20 by Thr or Ser, Gly23 by Asn, ILe24 by Asn, Phe36 by Asn, Asp37 by Asn, or Ser39 by Asn, or a combination of two or more of these substitutions.

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Brief Summary Text - BSTX (13):

Amino-acid residues responsible for N-glycosylation are Asn-X-Thr or Asn-X-Ser (amino-acid residues are indicated by the three-letter code and the X represents any amino-acid residues). Modified TCF lacking specific oligosaccharide chain(s) can be obtained by expressing the nucleotide sequence in which the codons for Asn; Ser or Thr are deleted or substituted with those for one of the other amino acids, in eukaryotic cells, preferably mammalian cells. Modified TCF which lacks specific oligosaccharide chains can be obtained by replacing the codons for Asn with those for other amino acids such as Gln, a substitution with little effects on the conformation of TCF. Gln is the most preferable amino acid which substitutes for Asn. Asp, Glu, His, Ser or Thr are also acceptable. Modified TCF lacking specific oligosaccharide chain(s) can be obtained by replacing the codons for Ser with those for other amino acids such as Ala, a substitution with little effects on the conformation of TCF. Ala is the most preferable amino acid which substitute for Ser. Pro, Gly or Asn are also acceptable. Modified TCF lacking specific oligosaccharide chain(s) can be obtained by deleting or replacing the codons for Thr to other amino acids. Ala is the most preferable amino acid which substitutes for Thr. Val, His or Asn are also acceptable.

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Vascular endothelial growth factor B, a novel growth factor for endothelial cells

(angiogenesis/endothelium/mitosis)

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ABSTRACT We have isolated and characterized a novel growth factor for endothelial cells, vascular endothelial growth factor B (VEGF-B), with structural similarities to vascular endothelial growth factor (VEGF) and placenta growth factor. VEGF-B was particularly abundant in heart and skeletal muscle and was coexpressed with VEGF in these and other tissues. VEGF-B formed cell-surface-associated disulfide-linked homodimers and heterodimerized with VEGF when coexpressed. Conditioned medium from transfected 293EBNA cells expressing VEGF-B stimulated DNA synthesis in endothelial cells. Our results suggest that VEGF-B has a role in angiogenesis and endothelial cell growth, particularly in muscle.

Angiogenesis, the sprouting of new capillaries from preexisting blood vessels, is of central importance in many biological processes. During embryonic development, vascularization of many organs and structures is achieved by angiogenesis from the larger vascular network formed by the growth and differentiation of both early embryonic and extraembryonic splanchnic mesoderm in a process called vasculogenesis (1). Furthermore, tissue regeneration and reorganization, as well as several pathological conditions including growth and metastasis formation of solid tumors, have been shown to be dependent on the formation of new blood vessels (2). The endothelial cell is the major cell type contributing to neovascularization. During angiogenesis, endothelial cells, which form the inner lining of blood vessels, undergo migration with concomitant proliferation and tube formation and participate in proteolytic degradation of the basement membrane and extracellular matrix. The specific mechanisms regulating angiogenesis are not fully understood, but several potential regulators of this process have been described (3). Vascular endothelial growth factor (VEGF) is an endothelial-cell-specific mitogen and has been shown to be a potent angiogenic factor (4). It exerts its effect through two endothelial receptor tyrosine kinases (RTKs) Flk-1/KDR (5-7) and Flt-1 (8, 9), which appear to play a pivotal role in regulation of endothelial cell growth and differentiation and in maintenance of the functions of the mature endothelium (10, 11). In addition, three other RTKs have been discovered that are specifically expressed in endothelial cells, Flt-4 (12-14), TIE, and TEK (15-17), of which TIE and TEK have been shown to be indispensable for the development of a proper vasculature (18, 19). Thus, endothelial cell growth factors have several attractive features as regulators of normal and pathological angiogenesis (4, 20-22). Placenta growth factor (PlGF) (23), which is a ligand for the Flt-1 RTK (24), is structurally related to VEGF, but its biological function remains obscure at present.

Both VEGF and PlGF are dimeric glycoproteins that are related in structure to platelet-derived growth factor A and B polypeptides (PDGF-A and -B), potent mitogens for smooth muscle cells, glia cells, and several other cell types (25). In this study, we have characterized a novel growth factor for endothelial cells VEGF-B,^{**} with structural homology to VEGF, PlGF, and the two PDGF polypeptides. Our results suggest that VEGF-B plays a role in vascularization of adult and embryonic tissues, in particular of muscle.

MATERIALS AND METHODS

Cloning and cDNAs Encoding Mouse and Human VEGF-B.

A cDNA clone termed pcif 2, encoding part of mouse VEGF-B, was isolated from a mouse embryonic day (E) 14.5 cDNA library cloned in the yeast expression vector pPC67 (26). A 0.9-kb *Sal* I-*Not* I insert in this cDNA clone was used to screen an adult mouse heart λ ZAP II cDNA library (Stratagene). Several positive clones were subcloned by *in vivo* excision into pBluescript SK(+) and the nucleotide sequences of the inserts were determined (Sequenase 2.0, United States Biochemical). To obtain cDNA clones encoding human VEGF-B, 10⁶ clones of a human fibrosarcoma cDNA library HT-1080 in λ gt11 (Clontech) were screened with the same insert. Among several positive clones, one, termed H.1, was analyzed and its nucleotide sequence was determined. Based on this sequence, two oligonucleotides were designed and used to amplify by reverse transcription-coupled PCR the whole coding region of human VEGF-B from oligo-(dT)-primed human erythroleukemia cell (HEL) RNA. The amplified product was cloned into the pCRII-vector of the TA cloning kit (Invitrogen) and the nucleotide sequence was determined. Standard molecular biology techniques were used throughout this work (27). The multiple amino acid sequence alignments and the phylogenetic analysis were done according to Hein (28) by using the PAM 250 distance table.

Northern Blot Analysis and *in Situ* Hybridization. Mouse and human multiple-tissue Northern blots (Clontech) were hybridized with a ³²P-labeled mouse VEGF-B probe (0.9-kb *Sal* I-*Not* I insert of clone pcif2). VEGF expression was analyzed with ³²P-labeled VEGF₁₆₅ cDNA as the probe (a gift from Daniel Connolly) (29). The hybridizations were carried out at 42°C in 50% (vol/vol) deionized formamide/5× SSC.

Abbreviations: VEGF, vascular endothelial growth factor; PDGF, platelet-derived growth factor; PlGF, placenta growth factor; RTK, receptor tyrosine kinase; HUVEC, human umbilical vein endothelial cell; bFGF, basic fibroblast growth factor; E, embryonic day; BCE, bovine capillary cell.

[†]B.O. and K.P. contributed equally to this work, as did K.A. and U.E.
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^{**}The sequences reported in this paper have been deposited in the GenBank data base (accession nos. U48800 and U48801 for mouse and human VEGF-B₁₆₇, respectively).

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pH 7.0/1% SDS/5× Denhardt's solution/denatured salmon sperm DNA (100 µg/ml). The filters were washed for two 30-min periods at 52°C in 2× SSC/0.5% SDS and exposed to Kodak XAR film with intensifying screens for 2–5 days at –70°C. *In situ* hybridization analysis of adult mouse tissues from CBA mice and of embryos derived from matings of CBA and NMR1 mice were carried out essentially as described (30). The RNA probes (a 383-bp antisense probe and a 169-bp sense probe) were generated from a linearized plasmid containing a 440-bp *Sal* I–*Sac* I fragment derived from the pcif2 cDNA clone. Radiolabeled RNA was synthesized by using T7 and SP6 RNA polymerases and ³²S-labeled UTP (Amersham). Alkaline hydrolysis of the probes was omitted. Hematoxylin was used for counterstaining. Control hybridizations with sense-strand, and RNase A-treated sections did not give signals above background.

Expression of VEGF-B and VEGF in 293EBNA Cells. cDNA inserts encoding human VEGF-B and human VEGF₁₆₅ were cloned into the pREP7 expression vector (Invitrogen). Human embryo kidney 293EBNA cells (expressing Epstein-Barr virus nuclear antigen 1) were transfected with expression plasmids by using calcium phosphate precipitation, and the cells were incubated for 48 h. Monolayers of cells were incubated in methionine- and cysteine-free medium for 30 min followed by labeling with [³⁵S]methionine and [³⁵S]cysteine (100 µCi/ml) (Promix, Amersham; 1 Ci = 37 GBq) in the same medium for 2 h. The labeling medium was replaced with normal medium without serum and labeled proteins were chased for 6 h. Heparin was included during the chase when indicated (100 µg/ml). Medium was collected after the chase period and cells were solubilized in 10 mM Tris-HCl, pH 7.5/50 mM NaCl/0.5% sodium deoxycholate/0.5% Nonidet P-40/0.1% SDS/aprotinin (0.1 unit/ml). Aliquots of the culture supernatants and the cell lysates were subjected to immunoprecipitation with specific antisera and analysis by SDS/PAGE. The anti-peptide antiserum to human VEGF-B was generated by immunizing rabbits with a branched 23-mer oligopeptide composed of the N-terminal region (amino acid residues SQPDAPGHQRKVVSWIDVYTRAT). The antiserum to human VEGF was from R & D Systems.

Mitogenic Assay for VEGF-B. Conditioned medium containing human VEGF-B and human VEGF₁₆₅ was collected from 293EBNA cells transfected with the appropriate expression vectors or with empty vector (mock) in the presence of heparin (1 µg/ml) 48 h after transfection. Second-passage human umbilical vein endothelial cells (HUVECs) were plated

into 96-well plates (4 × 10³ cells per well) in M-199 medium supplemented with 10% (vol/vol) fetal bovine serum and incubated for 24 h. Conditioned medium was diluted with the growth medium and cells were stimulated for 48 h. Fresh conditioned medium containing [³H]thymidine (Amersham; 10 µCi/ml) was added to the cells and stimulation was continued for another 48 h. Cells were washed with PBS and trypsinized and the incorporated radioactivity was determined by liquid scintillation counting. Bovine capillary endothelial (BCE) cells were seeded into 24-well plates and grown until confluence in minimal essential medium (MEM) supplemented with 10% fetal calf serum. Cells were starved in MEM supplemented with 3% fetal calf serum for 72 h, after which conditioned medium diluted into serum-free medium was added to the cells and the cells were stimulated for 24 h. [³H]Thymidine was included during the last 4 h of the stimulation (1 µCi/ml). Stimulations with basic fibroblast growth factor (bFGF) were carried out as above by using recombinant bFGF at 6 ng/ml (a gift from Andreas Sommer, Synergen, Boulder, CO). Cells were washed with PBS and lysed with NaOH, and incorporated radioactivity was determined by liquid scintillation counting.

RESULTS

Primary Structures of Mouse and Human VEGF-B. By using the yeast two-hybrid system for other purposes, we isolated a partial cDNA clone from a mouse E14.5 cDNA library encoding amino acid sequences similar to the VEGF family of growth factors. With this cDNA as a probe, several cDNA clones were isolated from an adult mouse heart cDNA library and from human fibrosarcoma and erythroleukemia tumor cell cDNAs. The mouse and human full-length cDNA clones encoded polypeptides of 188 amino acids containing an N-terminal hydrophobic putative signal sequence (Fig. 1). In analogy with VEGF (4), we propose that the signal peptidase cleavage site is located between Ala²¹ and Pro²². Hence, the processed VEGF-B polypeptides contained 167 amino acids. Mouse and human VEGF-B displayed 88% amino acid sequence identity and were highly basic, especially in their C-terminal regions. The amino acid replacements were predominantly found in the N- and C-terminal regions, while the central portions of the molecules were almost identical. Both human and mouse VEGF-B lacked the consensus sequence for N-linked glycosylation (NXT/S). Pairwise comparisons of the amino acid sequences showed that mouse VEGF-B is ~43%

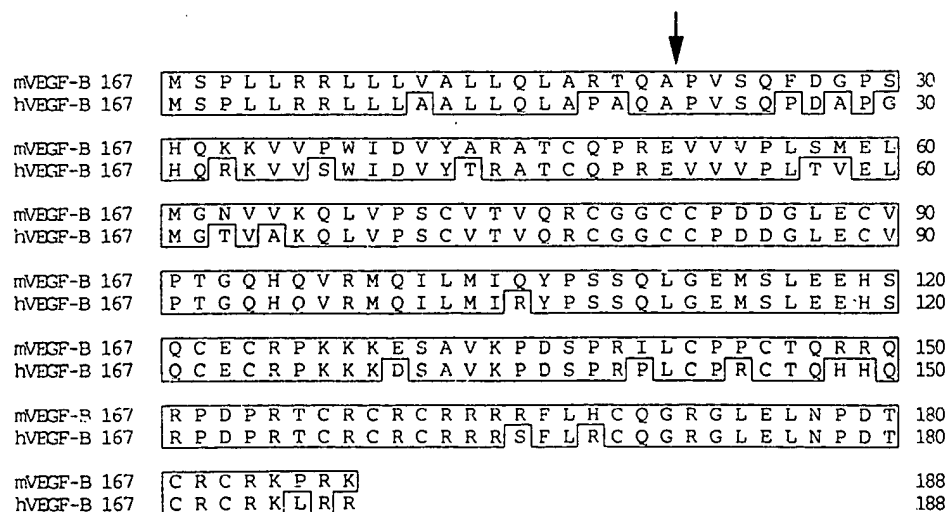


FIG. 1. Deduced amino acid sequences of mouse and human VEGF-B. Residues identical to mouse VEGF-B are boxed. The arrow indicates the putative cleavage site of the signal peptidase.

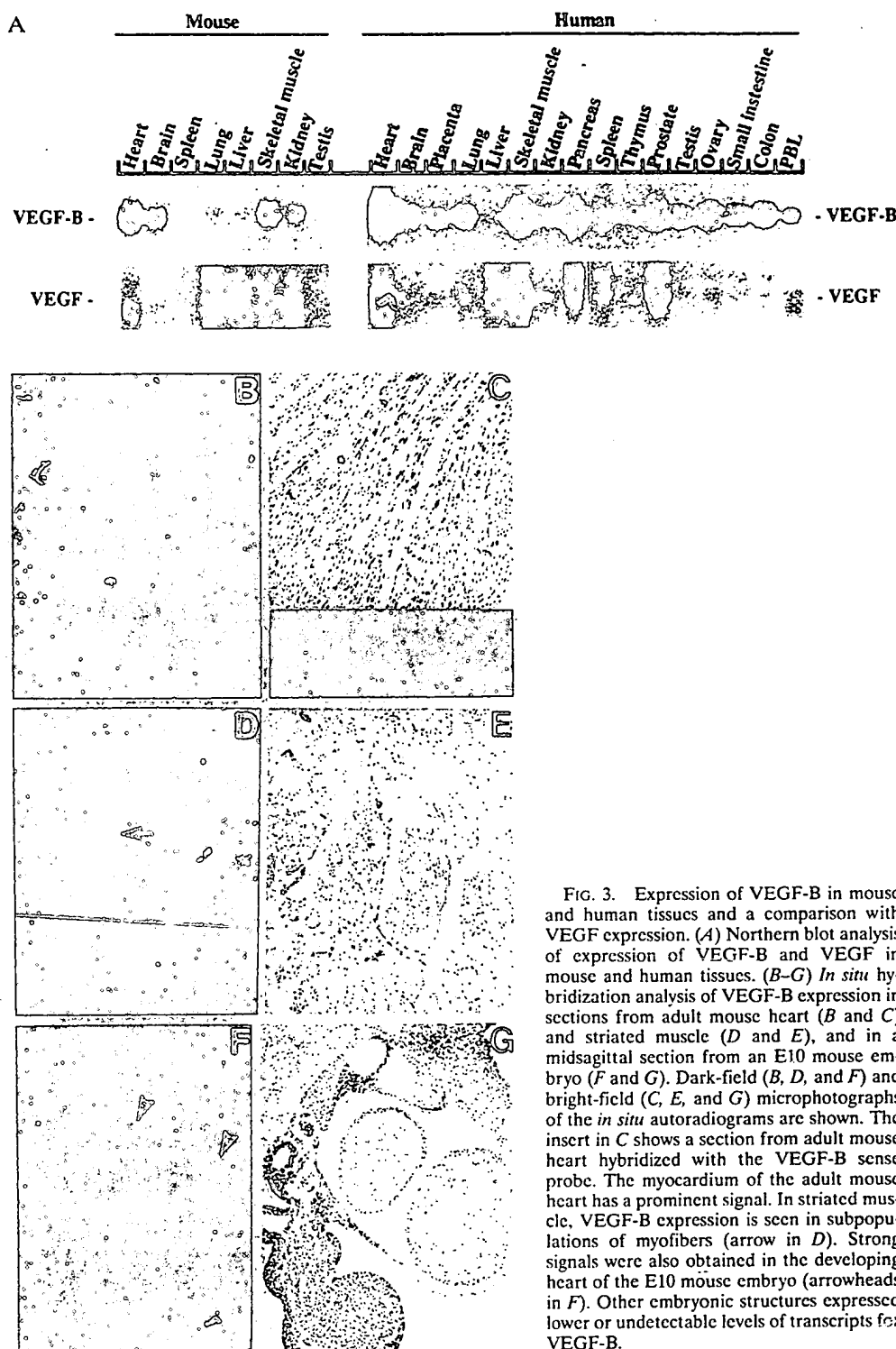


FIG. 3. Expression of VEGF-B in mouse and human tissues and a comparison with VEGF expression. (A) Northern blot analysis of expression of VEGF-B and VEGF in mouse and human tissues. (B-G) *In situ* hybridization analysis of VEGF-B expression in sections from adult mouse heart (B and C) and striated muscle (D and E), and in a midsagittal section from an E10 mouse embryo (F and G). Dark-field (B, D, and F) and bright-field (C, E, and G) microphotographs of the *in situ* autoradiograms are shown. The insert in C shows a section from adult mouse heart hybridized with the VEGF-B sense probe. The myocardium of the adult mouse heart has a prominent signal. In striated muscle, VEGF-B expression is seen in subpopulations of myofibers (arrow in D). Strong signals were also obtained in the developing heart of the E10 mouse embryo (arrowheads in F). Other embryonic structures expressed lower or undetectable levels of transcripts for VEGF-B.

factors, may act in a paracrine fashion to regulate endothelial cell function.

VEGF-B forms disulfide-linked dimers that are secreted but remain bound to cells or to the extracellular matrix and can be released by heparin or high salt treatments. This association is likely to be mediated by the C-terminal basic domain, as observed for the longer and highly basic splice variants of VEGF (4, 35, 36). The association of VEGF-B to cells or to the extracellular matrix may have several impor-

tant implications for regulation of its bioavailability and action in regulation of endothelial cell growth during embryonic development and in maintenance of the vasculature in adult tissues. Cell-associated VEGF-B may act as a local trophic factor and as a growth stimulus for endothelial cells by direct cell-cell interactions. This can be of particular significance in developing embryos and in contractile tissues, such as muscle, by providing spatial cues to outgrowing endothelial cells during establishment and maintenance of

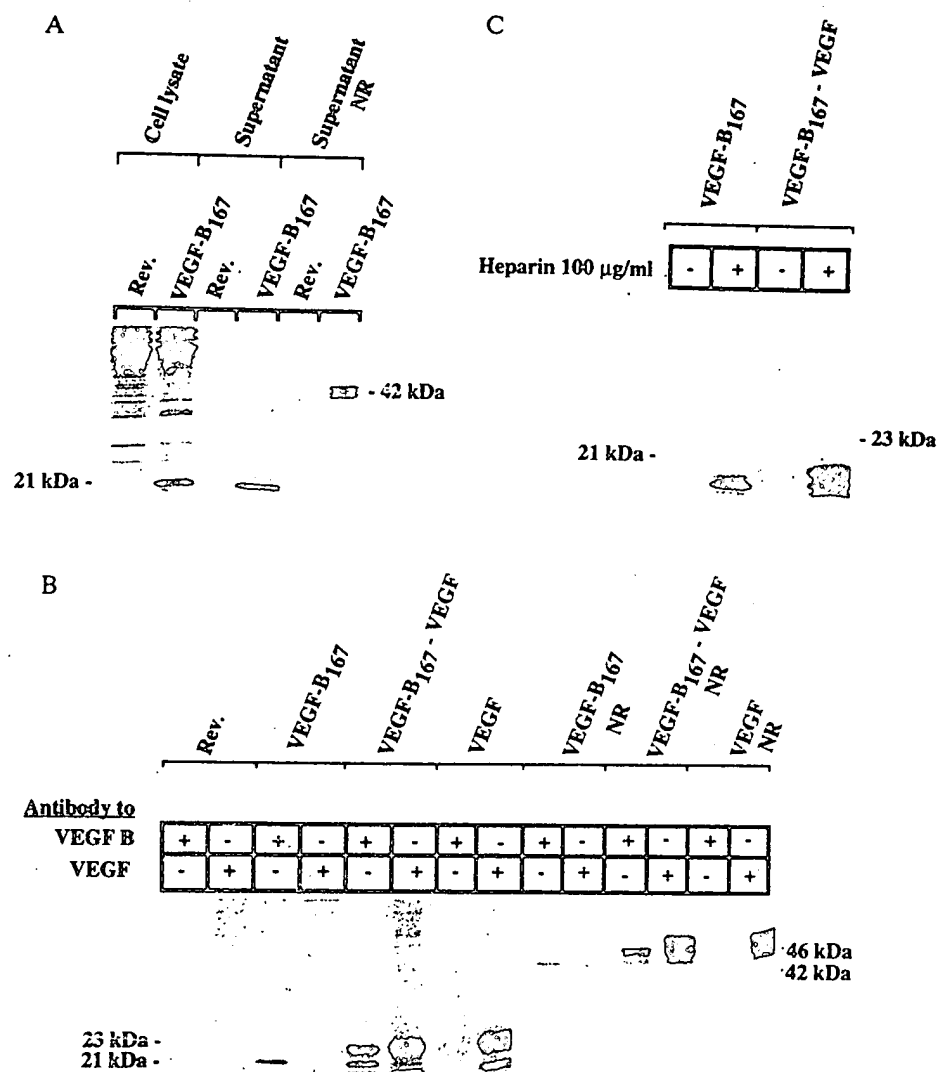


FIG. 4. Biochemical properties of VEGF-B and heterodimerization with VEGF. Human VEGF-B and human VEGF₁₆₅ were expressed, either separately or in combination, in human embryonic kidney 293EBNA cells by transient transfection and the cells were metabolically labeled. Culture supernatants and detergent-solubilized cell lysates were subjected to immunoprecipitation and SDS/PAGE analysis under reducing conditions unless otherwise indicated. (A) VEGF-B was expressed as 21-kDa protein in the transfected cells. As a control, cells were transfected with an expression vector containing the VEGF-B cDNA in reverse orientation (Rev.). By heparin treatment, VEGF-B was released from cells and found in the supernatant. Analysis of culture supernatants under nonreducing conditions (NR) showed that VEGF-B migrated as a 42-kDa species, indicating a dimeric structure. (B) VEGF-B and VEGF₁₆₅ were expressed in transfected 293EBNA cells, either separately or in combination. Anti-peptide antisera to VEGF-B or VEGF were used in immunoprecipitation analysis of culture supernatant from heparin-treated cells expressing VEGF-B, from cells coexpressing VEGF-B and VEGF₁₆₅, from cells expressing VEGF₁₆₅ alone, and from control cells (Rev.). Under nonreducing conditions (NR), the VEGF-B-VEGF heterodimers migrated as species of 42–46 kDa. (C) VEGF-B was expressed alone or in combination with VEGF. Culture supernatants from cells treated or untreated with heparin were subjected to immunoprecipitation with an anti-peptide antiserum to VEGF-B and analyzed by SDS/PAGE. The data show that VEGF-B homodimers and VEGF-B-VEGF heterodimers are released from cells by heparin.

the vascular tree. Alternatively, cell association may functionally inactivate VEGF-B by making it inaccessible for endothelial cells. Upon trauma or other injuries, it could be rapidly released and thus functionally activated. This latter possibility is attractive in light of the general slow turnover of endothelial cells in most tissues.

The ability of VEGF-B to heterodimerize with VEGF is consistent with the conservation of the eight cysteine residues involved in inter- and intramolecular disulfide bonding. Furthermore, the coexpression of VEGF-B and VEGF in many tissues suggests that VEGF-B-VEGF heterodimers occur naturally. The formation and cell association of such heterodimers may affect the formation of VEGF homodimers and

thus indirectly control release and bioavailability of VEGF. The formation of VEGF-B-VEGF heterodimers imply that cellular signals via these two growth factors are at least partly overlapping. VEGF was recently shown also to form heterodimers with PlGF (33). The formation of different heterodimeric complexes of these growth factors, their differential expression patterns during embryonic development and in adults, and different biochemical properties could provide a basis for a diverse array of regulatory signals for endothelial cells.

At least two RTKs, Flk-1/KDR and Flt-1, are involved in signaling mediated via VEGF and PlGF. Whether VEGF-B binds to the same RTKs or whether a novel and yet unidentified RTK is the receptor for VEGF-B remains to be eluci-

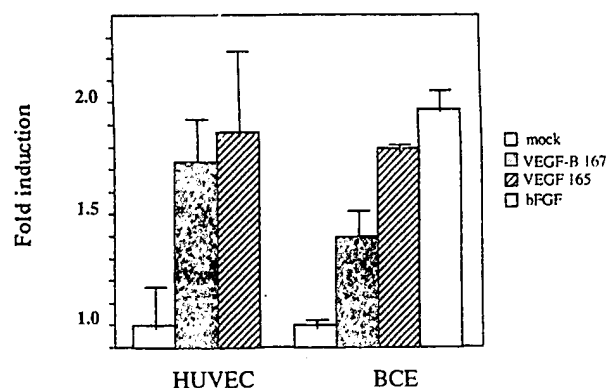


FIG. 5. VEGF-B induces $[^3\text{H}]$ thymidine incorporation into endothelial cells. Conditioned medium from 293EBNA cells transfected with expression vectors for VEGF-B, VEGF₁₆₅, or empty vector (mock) were diluted in respective media, applied to HUVECs and to BCE cells, and incorporation of $[^3\text{H}]$ thymidine was measured. As a positive control recombinant bFGF was added to BCE cells. The columns show fold induction of $[^3\text{H}]$ thymidine incorporation compared to basal activity induced by conditioned medium from mock-transfected cells. The bars show the mean \pm SD of parallel samples, and similar results were obtained in several independent experiments.

dated. However, with the molecular tools now available, a detailed analysis of the role of VEGF-B in establishing and maintaining normal and pathological vascularization and endothelial cell growth of tissues can be undertaken.

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